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# The influence of simulated global ocean acidification on the toxic effects of carbon nanoparticles on polychaetes

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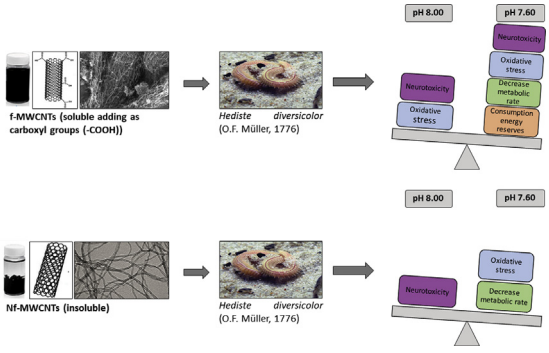
## HIGHLIGHTS

- Slighter aggregation and more suspended carbon nanomaterials in acidified seawater
- Under pH acidified both carbon nanomaterials generated greater oxidative stress in polychaetes.
- Functionalized carbon nanomaterials increased oxidative stress and neurotoxicity under both pHs.
- Ocean acidification may cause a higher risk of carbon nanomaterials to marine ecosystems.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Ocean acidification events are recognized as important drivers of change in biological systems. Particularly, the impacts of acidification are more severe in estuarine systems than in surface ocean due to their shallowness, low buffering capacity, low salinity and high organic matter from land drainage. Moreover, because they are transitional areas, estuaries can be seriously impacted by a vast number of anthropogenic activities and in the last decades, carbon nanomaterials (CNMs) are considered as emerging contaminants in these ecosystems. Considering all these evidences, chronic experiment was carried out, trying to understand the possible alteration on the chemical behaviour of two different CNMs (functionalized and pristine) in predicted climate change scenarios and consequently, how these alterations could modify the sensitivity of one the most common marine and estuarine organisms (the polychaeta *Hediste diversicolor*) assessing a set of biomarkers related to polychaetes oxidative status as well as the metabolic performance and neurotoxicity. Our results demonstrated that all enzymes worked together to counteract seawater acidification and CNMs, however oxidative stress in the exposed polychaetes to both CNMs, especially under ocean acidification conditions, was enhanced. In fact, although the antioxidant enzymes tried to cope as compensatory response of cellular defense systems against oxidative stress, the synergistic interactive effects of pH and functionalized CNMs indicated that acidified pH significantly increased the oxidative damage (in terms of lipid peroxidation) in the cotaminated organisms. Different responses were observed in organisms submitted to pristine CNMs under pH control, where the lipid peroxidation did not increase along with the increasing exposure concentrations. The present results further demonstrated

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neurotoxicity caused by both CNMs, especially noticeable at acidified conditions. The mechanism of enhanced toxicity could be attributed to slighter aggregation and more suspended NMs in acidified seawater (as demonstrated by the DLS analysis). Therefore, ocean acidification may cause a higher risk of CNMs to marine ecosystems.

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## 1. Introduction

As a consequence of human activity the carbon dioxide ($CO_2$) emissions increased at the beginning of the industrial age and are now enhancing annually the atmospheric concentration of $CO_2$ by 1–2 ppm by volume (ppmv) (Davis, 2017), resulting in global warming and ocean acidification, actually recognized as important drivers of biological systems (Fabry et al., 2008). Considering that one-third of the anthropogenic $CO_2$ produced in the past 200 years has been taken up by the oceans (Sabine et al., 2004), the direct effect of $CO_2$ on ocean chemistry may affect marine biota. In this regard, most of the studies are focused on marine calcifying organisms, such as corals, mollusks, echinoderms and crustaceans, as a consequence of reduction of carbonate ions that are necessary to produce $CaCO_3$ for the construction of their shells and skeletons (Feely et al., 2004). However, other physiological and biochemical indices appear to be correlated with the capacity for acid–base tolerance, including survival, growth, development, metabolism of marine non-calcyfing organisms under elevated $pCO_2$ (Feely et al., 2004). Nevertheless, despite the increasing number of studies related to the effects of the ocean acidification on the ocean ecosystem, less is known on the effects of acidification on estuarine ecosystems (Glaspie et al., 2018). The impacts of estuarine acidification are more severe than surface ocean due to its shallowness, low buffering capacity, low salinity and high organic matter from land drainage (Miller et al., 2009; Glaspie et al., 2018).

Furthermore, due to their location, estuaries can also be seriously impacted by different anthropogenic activities (McLusky et al., 2004), which in turn can be influenced by climate change related factors such as acidification. Estuaries can easily become polluted due to industrial waste and agricultural and horticultural run-off such as heavy metals and polycyclic aromatic hydrocarbons (PAHs), nutrients, oil spills (https://oceanservice.noaa.gov/education/kits/estuaries/estuaries09_humandisturb.html), and in the last decades, U.S. Environmental Protection Agency (EPA) Federal Facilities Restoration and Reuse Office (FFRRO) have provided a brief summary of nanomaterials (NMs) as emerging contaminants in the estuarine ecosystem. Within the NMs, carbon nanomaterials (CNMs) have a unique place in nanoscience due to their expecional properties, including strong adsorption capacity, chemical stability, and high mechanical strength as well as easy modification, resulting in their wide use in different areas as composite materials, energy storage and conversion, sensors, drug delivery, field emission devices and nanoscale electronic components (Yan et al., 2016). As a consequence, coastal environments such as estuaries are the expected destination of CNMs, through industrial discharges and municipal effluents (Wang et al., 2014), with known impacts on inhabiting organisms (Andrade et al., 2018; De Marchi et al., 2017a, 2017b, 2018a, 2018b, 2018c; Boncel et al., 2015; Galloway et al., 2010; Baun et al., 2008). Nevertheless, the interactive effects of these emerging contaminants with acidification in estuarine systems needs to be further clarified as one of the first steps towards the evaluation of the effects on inhabiting organisms (Ferry et al., 2009).

Therefore, we conducted a chronic expsoure (28 days) to understand the possible alteration on the chemical behaviour and toxicity of two different CNMs as well as the possible alteration on the sensitivity of a common estuarine species (the polychaeta *Hediste diversicolor*) to CNMs due to seawater acidification. For this, biomarkers related to polychaetes oxidative status as well as metabolic performance and potential neurotoxicity were assessed.

## 2. Materials and methods

### 2.1. Model species

Polychaetes are well represented in most marine and estuarine environments, both in terms of number of individuals and species, and they typically represent a significant percentage of the total macrofaunal diversity (Hutchings, 1998). The common ragworm *Hediste diversicolor* (O.F. Müller, 1776) is one of the most important and abundant species inhabiting intertidal mudflats of estuaries and shallow water bodies of the earth (Arias et al., 2016; Dağli et al., 2005) playing important ecological (Thit et al., 2015; Catalano et al., 2012) and economic (Costa et al., 2006; Cunha et al., 2005) roles. Moreover this species is considered a good bioindicator due to its burrowing capacity and dietary behaviour (Gillet et al., 2008; Durou et al., 2007; Amiard et al., 2007). In particular, this species has been commonly used to assess pollution impacts due to metals (Bouraoui et al., 2010; Pook et al., 2009; Burlinson and Lawrence, 2007; Moreira et al., 2006), polycyclic aromatic hydrocarbons (Catalano et al., 2012; Sun and Zhou, 2008), pharmaceuticals (Pires et al., 2016; Maranho et al., 2014) and more recently to NMs (De Marchi et al., 2017a, 2018a; Monserrat et al., 2017; Thit et al., 2015; Bour et al., 2015; Buffet et al., 2014a, 2014b; Cong et al., 2014; Moschino et al., 2014; Matranga and Corsi, 2012; Cong et al., 2011).

### 2.2. Collection and acclimation period

*H. diversicolor* specimens were collected from the Mira channel in Ria de Aveiro, a shallow coastal lagoon situated on the north-west Atlantic coast of Portugal and organisms with similar weight (0.53 ± 0.2 g) were used to prevent differences on biochemical responses.

Organisms were allowed to acclimate (2 weeks) in 5 different aquaria (20 l each; 50 specimens *per* aquarium) filled with a mixture of fine and medium sediment from the sampling area (see specification in De Marchi et al., 2018a) and artificial seawater (salinity 28), made by the addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water, one day prior to utilization. Temperature was kept at 18 ± 1 °C, photoperiod of 12 h light: 12 h dark, pH of the sampling area (8.00 ± 1 units) and constant aeration. During this period, specimens were fed *ad libitum* with commercial fish food every two-three days (48.6% protein and 7.7% fat) (De Marchi et al., 2018a).

### 2.3. Experimental set up

#### 2.3.1. pH variations

To simulate mid-twentieth century and future seawater conditions organisms were exposed for 28 days to 2 pH levels (control-8.00 and acidified-7.60), each one acting alone and combined with two environmental relevant concentrations of NMs. Lowered pH was set to 7.60 to give a 0.4 pH units' reduction relative to control (pH 8.00, mean pH level at estuarine waters), a pH value selected considering the predicted scenario of climate change for 2100 (IPCC, 2013). Low pH exposure was obtained by directly and automatically diffusing $CO_2$ into aquaria. Individual aquarium pH levels were continuously monitored and controlled using a pH Stat system (Aquamedic). Immediately before the starting of the experiment, the acidified pH was progressively decreased to avoid additional osmotic stress to the polychaetes. During the exposure period every week water was renewed and pH conditions re-established

with acidified water previously prepared (one day prior the use) for the low pH conditions. Immediately after and before water renewal, water samples (50 mL) were collected for the physicochemical parameters of the used water. At the same time, pH, temperature (T (°C)), salinity (S) and determined total alkalinity (TA) were analyzed at each of the tested conditions. Subsequently, partial $CO_2$ pressure ($pCO_2$), bicarbonate ($HCO_3^-$), carbonate ion concentrations ($CO_3^{2-}$), saturation states of calcite (ΩCal) and aragonite (ΩAg) were calculated with CO2SYS software (Table 1).

*2.3.2. Contaminants description*

The CNMs used in the present study corresponded to two types of Multi Walled Carbon Nanotubes (MWCNTs): pristine MWCNTs (Nf-MWCNTs) (MWCNTs: NC7000 series, http://www.nanocyl.com) and chemically functionalized MWCNTs, by introducing polar groups such as carboxyl groups (-COOH) (f-MWCNTs) (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com), both at the environmental relevant concentrations of 0.01 and 0.10 mg/L. All the technical data of both materials are specified in Table 2.

The selection of these two CNTs was based on their different physical and chemical properties and different behaviour in the water media (aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution) (Arndt et al., 2013). Carboxylated CNMs are more stable in salt water media in comparison to pristine MWCNTs as a consequence of their oxidation process which introduces oxygen-containing groups on the surface. These groups ionize in water charging the oxygen atoms negatively and in aqueous phase the electrostatic repulsive forces between negative surface charges of the oxygen-containing groups can lead to stability of oxidized CNTs in the water column increasing the availability of these materials for the organisms (Peng et al., 2009).

Moreover, the selection of the CNMs was based on their wide range of industrial applicabilities (see specifications of both used materials on MWCNTs-COOH: TNMC1 series, http://www.timesnano.com; MWCNTs: http://www.nanocyl.com/product/nc7000/), that will certainly increase the probability of presence in estuarine environments.

Although, to our knowledge, the environmentally relevant concentrations (ERCs) of CNTs in water, based on a stochastic/probabilistic material-flow computer model, are in the μg/L or ng/L range (Sun et al., 2016), the predicted environmental concentrations (PECs) of CNTs in aqueous systems reported from the most recent literature (Nouara et al., 2013; Zhang et al., 2017) were projected to approximately 1–1000 μg/L. Thus, the exposure concentrations of both materials were selected considering this range of concentrations. Both CNMs were weighed (stock solution of 50 mg/L) and suspended in seawater. To promote stable suspension of both materials in the water column (Hwang et al., 2007), the Nf-MWCNTs was sonicated for 1 h using 30 Hz ultrasound bath (IKA Labortechnik IKASONIC U50), while the f-MWCNTs, due to the presence of carboxyl groups was sonicated for few minutes (Shahnawaz et al., 2010). The concentrations of both CNMs were re-established weekly after complete water renewals to ensure the same exposure concentrations during the experiment. The added MWCNT materials were homogenously dispersed in the seawater using one submersible circulation pump *per* aquarium, increasing both CNMs mass suspended in the water column (Vonk et al., 2009).

*2.4. Analyses of MWCNTs in water*

The average size distribution measured by dynamic light scattering (DLS) and the polydispersity index (PDI) of both MWCNT materials suspended in artificial seawater at different pH levels and exposure times were analyzed (control pH-Table 3; acidify pH-Table 4). In the present work, DLS measurements were carried out to obtain data regarding the tendency to aggregate and the settling behaviour of suspended CNT materials in aqueous media. Immediately before the water renewal, water samples (50 mL) *per* replicate were collected for the average size distribution measured by dynamic light scattering (DLS) and the polydispersity index (PDI) of both MWCNT materials suspensions at different pH levels and exposure times (T0: time zero, immediately after the dispersion of the materials in a water medium; T7: water samples collected after 1 week of exposure; T14: water samples collected after 2 weeks of exposure; T21: water samples collected after 3 weeks of exposure and T28: samples collected after 4 weeks of exposure). Measurements were performed on 1000 μL of suspension in four samples *per* replicate (three replicates *per* condition), and five analyses per sample performed by DLS using a DelsaTM NanoC Particle Size Analyzer (Beckman Coulter). Each analysis was carried out by performing 120 acquisitions. Due to the inherent heterogeneity and colloidal instability of the analyzed samples, DLS analyses were repeated several times to ensure reproducible results. Size distributions were obtained by analyzing the autocorrelation functions through the Contin algorithm which is particularly ideal for polydisperse and multimodal systems (Varenne et al., 2016). The cumulant method was used to obtain information on the particles average hydrodynamic radii and on the PDI (Tardani and Mesa, 2015).

*2.5. Biochemical parameters*

At the end of the experimental period (28 days), three pools of organisms, each corresponding to three whole body individuals (9 individuals in total *per* aquarium, 27 *per* condition) were pulverized with liquid nitrogen, divided in 0.2 g fresh weight (FW) aliquots, and used for biochemical analyses. Extractions were performed with specific buffers to determine: I) energy reserves and metabolic capacity (protein (PROT) and glycogen (GLY) contents, electron transport system (ETS) activity); II) indicators of oxidative stress (lipid peroxidation (LPO) levels, catalase (CAT), superoxide dismutase (SOD), Glutathione reductase (GR) activities) and III) neurotoxicity (Acetylcholinesterase (AChE)

**Table 1**
Carbonate system physicochemical parameters for pH experiments (mean ± SD; n = 3). Measured pH, Temperature (T (°C)), Salinity (S) and determined total alkalinity (TA) from weekly water sampling of pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH 8.00 and acidified pH 7.60. Partial $CO_2$ pressure ($pCO_2$), bicarbonate ($HCO_3^-$), carbonate ion concentrations ($CO_3^{2-}$), saturation states of calcite (ΩCal) and aragonite (ΩAg), calculated with CO2SYS software.

| | | | pH | T (°C) | Salinity | TA (μmol/kg) | $pCO_2$ (μatm) | $HCO^{3-}$ (μmol/kgSW) | $CO_3^{2-}$ (μmol/kgSW) | ΩCal | ΩAra |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | pH 8.00 | Nf | 8.09 ± 0.03 | 16.3 ± 2.63 | 31.33 ± 0.58 | 2022.54 ± 20.88 | 442.53 ± 46.82 | 1728.88 ± 12.47 | 116.41 ± 4.43 | 2.85 ± 0.11 | 1.82 ± 0.08 |
| | | f | 8.09 ± 0.03 | 16.3 ± 2.63 | 31.33 ± 0.58 | 2022.54 ± 20.88 | 442.53 ± 46.82 | 1728.88 ± 12.47 | 116.41 ± 4.43 | 2.85 ± 0.11 | 1.82 ± 0.08 |
| | pH 7.60 | Nf | 7.61 ± 0.04 | 16.3 ± 2.63 | 31.34 ± 0.51 | 1821.96 ± 55.24 | 1318.91 ± 128.62 | 1721.08 ± 60.99 | 39.08 ± 6.69 | 0.05 ± 0.16 | 0.61 ± 0.11 |
| | | f | 7.61 ± 0.04 | 16.3 ± 2.63 | 31.34 ± 0.51 | 1821.96 ± 55.24 | 1318.91 ± 128.62 | 1721.08 ± 60.99 | 39.08 ± 6.69 | 0.05 ± 0.16 | 0.61 ± 0.11 |
| 0.01 | pH 8.00 | Nf | 8.08 ± 0.04 | 16.3 ± 2.63 | 30.78 ± 0.69 | 2066.00 ± 72.32 | 471.19 ± 42.54 | 1779.09 ± 79.73 | 114.52 ± 11.96 | 2.81 ± 0.28 | 1.79 ± 0.19 |
| | | f | 8.10 ± 0.08 | 16.3 ± 2.63 | 30.89 ± 0.51 | 1888.82 ± 57.90 | 441.08 ± 78.91 | 1610.90 ± 38.74 | 108.81 ± 9.63 | 2.67 ± 0.24 | 1.70 ± 0.14 |
| | pH 7.60 | Nf | 7.65 ± 0.02 | 16.3 ± 2.63 | 31.33 ± 0.67 | 1608.01 ± 322.39 | 1225.99 ± 271.34 | 1521.51 ± 30.1 | 32.71 ± 9.69 | 0.80 ± 0.24 | 0.51 ± 0.16 |
| | | f | 7.63 ± 0.03 | 16.3 ± 2.63 | 31.44 ± 0.19 | 2427.05 ± 53.90 | 1701.73 ± 16.70 | 2296.54 ± 40.59 | 53.62 ± 7.30 | 1.31 ± 0.18 | 0.84 ± 0.12 |
| 0.10 | pH 8.00 | Nf | 8.11 ± 0.01 | 16.3 ± 2.63 | 30.56 ± 0.51 | 2045.95 ± 26.53 | 429.37 ± 47.62 | 1744.18 ± 28.43 | 120.36 ± 8.65 | 2.96 ± 0.22 | 1.89 ± 0.14 |
| | | f | 8.12 ± 0.05 | 16.3 ± 2.63 | 31.00 ± 0.00 | 2249.87 ± 32.24 | 463.81 ± 66.34 | 1914.82 ± 43.44 | 135.94 ± 11.51 | 3.33 ± 0.29 | 2.13 ± 0.19 |
| | pH 7.60 | Nf | 7.62 ± 0.01 | 16.3 ± 2.63 | 30.89 ± 0.38 | 1440.85 ± 32.24 | 1099.39 ± 60.28 | 1361.04 ± 24.18 | 29.19 ± 4.85 | 0.71 ± 0.12 | 0.46 ± 0.08 |
| | | f | 7.63 ± 0.03 | 16.3 ± 2.63 | 31.22 ± 0.38 | 2102.78 ± 57.90 | 1478.93 ± 134.04 | 1987.70 ± 60.53 | 46.02 ± 4.86 | 1.13 ± 0.12 | 0.72 ± 0.08 |

**Table 2**
Characterization of the powder form of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs).

| | Diameter (nm) | Length (um) | Carbon purity (%) | Surface area ($m^2$/g) | Amorphous carbon (mol%) | -COOH (wt%) |
|---|---|---|---|---|---|---|
| Nf-MWCNTs | 9.5 | 1.5 | 90 | 250–300 | [a] | – |
| f-MWCNTs | 2-5 | 10–30 | 98 | 400 | 8–10 | 3.86 |

[a] Pyrolytically deposited carbon on the surface of MWCNTs.

activity). The methodologies used to perform each specific biomarker are described in detail in De Marchi et al. (2018a, 2018c).

### 2.6. Data analysis

PERMANOVA+ add-on in PRIMER v6 was used as per mutational multivariate analysis of variance of the results regarding all the biochemical responses. A one-way hierarchical design was followed in this analysis. The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. Significant differences were observed using main test and consequently pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. The null hypotheses tested were: I) no significant impacts on polychaetes due to MWCNT material concentrations, regardless the pH tested levels; II) no effects of pH levels on the toxicity of MWCNT materials; III) no effects of pH variations on the sensitivity of polychaetes to MWCNT materials. For this we verified if: A) for each biomarker and for each pH variation, no significant differences existed between both MWCNT exposure concentrations (0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters in all the graphics: uppercase and regular letters for Nf-MWCNT at pH 8.00; lowercase and regular letters for Nf-MWCNTs pH 7.60; uppercase bold and italic letters for f-MWCNT at pH 8.00; lowercase, bold and italic letters for f-MWCNT at pH 7.60; B) for each biomarker and for pH variation and exposure concentration, no significant differences exist between MWCNT materials (Nf and f-MWCNTs). Significant differences ($p \leq 0.05$) between f-MWCNT and Nf-MWCNTs within each pH at each exposure concentration were represented with bold asterisks (*) in the Table 5; C) for each biomarker and for each MWCNTs material and exposure concentration, no significant differences exist between pH variation. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT materials and exposure concentration were represented with bold asterisks (*) in all the graphics.

## 3. Results

### 3.1. Analyses of MWCNTs in water

In the Tables 3 and 4 are reported the average size distribution and the polydispersity index (PDI) of Nf-MWCNT and f-MWCNT suspensions analyzed in each exposure concentrations (0.01 and 0.10 mg/L) under pH control (8.00) and acidify (7.60) respectively, at different exposure periods: T0: time zero, immediately after the dispersion of CNM materials in a water medium; T7: water samples collected after 1 week of exposure; T14: water samples collected after 2 weeks of exposure; T21: water samples collected after 3 weeks of exposure and T28: samples collected after 4 weeks of exposure.

At time 0 (T0), in the water samples collected at pH 8.00 the mean size of dispersed Nf-MWCNTs was largely higher than that of f-MWCNTs. DLS analysis did not evidence the presence of suspended particles at time 21 and 28 (T21 and T28) indicating the settlement and/or uptake of the material.

The mean size of dispersed Nf-MWCNTs water samples collected at T0 as well as at the other exposure times under pH 7.60, was largely higher than f-MWCNTs. Moreover, f-MWCNTs suspended at 0.10 mg/L

**Table 3**
The average size distribution (nm) and the polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analyzed in each exposure concentration (0.01 and 0.10 mg/L) under pH control 8.00 and at different exposure periods: T0: time zero, immediately after the dispersion of CNM materials in a water medium; T7: water samples collected after 1 week of exposure; T14: water samples collected after 2 weeks of exposure; T21: water samples collected after 3 weeks of exposure and T28: samples collected after 4 weeks of exposure.

| | Size (nm) | PDI | Size (nm) | PDI |
|---|---|---|---|---|
| Samples | Nf-MWCNTs | | f-MWCNTs | |
| *T0* | | | | |
| 0.01 mg/L | 1863.1 | 1.26 | I.d. | – |
| 0.10 mg/L | 5428.0 | 2.23 | 2545.1 | 1.13 |
| *T7* | | | | |
| 0.01 mg/L | I.d. | – | 5501.2 | 1.18 |
| 0.10 mg/L | 3217.4[a] | 1.39 | I.d. | – |
| *T14* | | | | |
| 0.01 mg/L | 8603.7[a] | 4.87 | 2344.9[a] | 1.38 |
| 0.10 mg/L | 1381.0[b] | 1.25 | I.d. | – |
| *T21* | | | | |
| 0.01 mg/L | I.d. | – | I.d. | – |
| 0.10 mg/L | I.d. | – | 1930.5[a] | 0.88[c] |
| *T28* | | | | |
| 0.01 mg/L | I.d. | – | I.d. | – |
| 0.10 mg/L | I.d. | – | I.d. | – |

I.d.: "Invalid data" (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
[a] Sample contaminated with sand grains and macroscopic blackish aggregates.
[b] Sample contaminated with sand grains and macroscopic particulates.
[c] Sample contaminated with macroscopic blackish aggregates.

**Table 4**
The average size distribution and the polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analyzed in each exposure concentrations (0.01 and 0.10 mg/L) under acidified pH 7.60 and at different exposure periods: T0: time zero, immediately after the dispersion of CNM materials in a water medium; T7: water samples collected after 1 week of exposure; T14: water samples collected after 2 weeks of exposure; T21: water samples collected after 3 weeks of exposure and T28: samples collected after 4 weeks of exposure.

| | Size (nm) | PDI | Size (nm) | PDI |
|---|---|---|---|---|
| Samples | Nf-MWCNTs | | f-MWCNTs | |
| *T0* | | | | |
| 0.01 mg/L | 1431.3 | 0.69 | I.d. | – |
| 0.10 mg/L | 3713.4 | 2.10 | 2116.1 | 0.97 |
| *T7* | | | | |
| 0.01 mg/L | Not supplied sample | – | I.d. | – |
| 0.10 mg/L | 5411.8 | 2.07 | 3116.0 | 1.23 |
| *T14* | | | | |
| 0.01 mg/L | Not supplied sample | – | I.d. | – |
| 0.10 mg/L | Not supplied sample | – | 4353.9 | 1.61 |
| *T21* | | | | |
| 0.01 mg/L | Not supplied sample | – | 2158.1[a] | 1.23 |
| 0.10 mg/L | I.d. | – | 2768.1[b] | 1.26 |
| *T28* | | | | |
| 0.01 mg/L | I.d. | – | I.d. | – |
| 0.10 mg/L | 3893.8 | 1.26 | 2818.2[c] | 1.53 |

I.d.: "Invalid data" (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
[a] Sample contaminated with sand grains and macroscopic blackish aggregates.
[b] Sample contaminated with sand grains and macroscopic particulates.
[c] Sample contaminated with macroscopic blackish aggregates.

**Table 5**
Effect on oxidative stress biomarkers (PROT, GLY, ETS, LPO, CAT, SOD, GR, AChE) in *Hediste diversicolor* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH 8.00 and acidified pH 7.60. Significant differences ($p \leq 0.05$) between f-MWCNT (F) and Nf-MWCNTs (NF) within each pH level at each exposure concentration were represented with asterisks (*).

| | | | PROT | GLY | ETS | LPO | CAT | SOD | GR | AChE |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | pH 8.00 | Nf | 53.9 ± 18.4 | 12.6 ± 2.9 | 69.6 ± 6.7 | 8.5 ± 2.8 | 47.5 ± 7.3 | 2.8 ± 0.7 | 0.7 ± 0.07 | 3.5 ± 0.3 |
| | | f | 53.9 ± 18.4 | 12.6 ± 2.9 | 69.6 ± 6.7 | 8.5 ± 2.8 | 47.5 ± 7.3 | 2.8 ± 0.7 | 0.7 ± 0.07 | 3.5 ± 0.3 |
| | pH 7.60 | Nf | 73.8 ± 3.1 | 14.2 ± 2.9 | 64.0 ± 6.7 | 8.6 ± 0.3 | 46.7 ± 7.7 | 2.2 ± 0.7 | 0.6 ± 0.07 | 2.6 ± 0.2 |
| | | f | 73.8 ± 3.1 | 14.2 ± 2.9 | 64.0 ± 6.7 | 8.6 ± 0.3 | 46.7 ± 7.7 | 2.2 ± 0.7 | 0.6 ± 0.07 | 2.6 ± 0.2 |
| 0.01 | pH 8.00 | Nf | 63.5 ± 24.6 | 13.2 ± 3.9 | 48.7 ± 18.4 | 9.6 ± 2.9 | 54.1 ± 9.7 | 4.9 ± 1.5* | 0.9 ± 0.10* | 1.4 ± 0.2* |
| | | f | 53.5 ± 9.5 | 10.3 ± 0.3 | 68.7 ± 16.5 | 9.7 ± 4.2 | 46.7 ± 9.2 | 2.9 ± 0.6 | 0.6 ± 0.06 | 3.1 ± 0.4 |
| | pH 7.60 | Nf | 95.5 ± 23.1* | 11.8 ± 1.8* | 64.2 ± 9.2 | 10.7 ± 1.5 | 59.6 ± 4.4 | 4.1 ± 1.6 | 0.6 ± 0.1 | 3.0 ± 0.3 |
| | | f | 60.6 ± 13.7 | 14.5 ± 1.8 | 54.0 ± 6.1 | 11.3 ± 2.0 | 52.2 ± 6.6 | 3.7 ± 1.4 | 0.7 ± 0.09 | 2.7 ± 0.8 |
| 0.10 | pH 8.00 | Nf | 51.0 ± 15.2 | 13.2 ± 4.0 | 72.3 ± 20.6 | 10.2 ± 2.2* | 53.9 ± 2.9 | 5.4 ± 0.9 | 0.6 ± 0.1 | 2.3 ± 0.5* |
| | | f | 42.3 ± 13.3 | 10.9 ± 1.7 | 84.6 ± 4.8 | 13.3 ± 2.6 | 49.1 ± 2.5 | 4.2 ± 0.5 | 0.6 ± 0.08 | 1.5 ± 0.3 |
| | pH 7.60 | Nf | 94.3 ± 15.8 | 18.6 ± 0.9 | 46.6 ± 10.8 | 12.7 ± 3.0* | 55.6 ± 8.0 | 4.0 ± 1.3 | 0.9 ± 0.1 | 3.5 ± 0.6* |
| | | f | 73.9 ± 26.0 | 17.9 ± 3.0 | 57.9 ± 5.7 | 17.7 ± 4.5 | 55.8 ± 3.8 | 5.7 ± 1.3 | 0.8 ± 0.1 | 1.2 ± 0.7 |

were found to agglomerate and remain dispersed in the medium until 28 days.

### 3.2. Mortality and biochemical parameters

No mortality was recorded after 28 days of exposure.

All the results of biochemical parameters were discussed considering: I) the effects of exposure concentrations of both MWCNT materials maintained under both pH variations; II) the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both pH levels for each exposure concentration; III) the effects of both pH levels in organisms exposed to both MWCNT materials in each exposure concentration.

#### 3.2.1. Energy reserves and metabolic capacity

*3.2.1.1. Protein (PROT) content.* Considering the effects of exposure concentrations on organisms PROT content, in individuals exposed to Nf-MWCNTs under both pH variations no significant differences were observed among exposure concentrations. A similar trend was observed in *H. diversicolor* exposed to f-MWCNTs under pH control, while a significantly concentration-depended was recorded when organisms were submitted to low pH in comparison to control pH (Fig. 1A).

When comparing *H. diversicolor* exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under pH 7.60, showing higher PROT content in individuals contaminated with Nf-MWCNTs (Table 5).

Significant differences between pH levels were observed in PROT content when organisms were exposed to 0.10 mg/L of f-MWCNTs, showing higher content in individuals maintained at pH 7.60 in comparison to pH 8.00 (Fig. 1A).

*3.2.1.2. Glycogen (GLY) content.* No significant differences in terms of GLY content were observed among Nf-MWCNT exposure concentrations in individuals mainteined under pH control, while under pH 7.60 polychaetes exposed to 0.10 mg/L showed significantly higher content in comparison to the remaining concentrations. In individuals exposed to f-MWCNTs under both pH levels no significant differences were observed among all exposure concentrations (Fig. 1B).

When comparing organisms exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under pH 7.60, with higher GLY content in individuals contaminated with f-MWCNTs (Table 5).

Significant differences between pH levels were observed in GLY content when organisms were exposed to 0.01 mg/L of f-MWCNTs, and at 0.10 mg/L of both MWCNT materials, showing in all cases higher content in individuals under pH 7.60 in comparison to pH 8.00 (Fig. 1B).

*3.2.1.3. Electron transport system (ETS) activity.* Considering the effects of exposure concentrations, results of ETS activity in *H. diversicolor* showed that for Nf-MWCNT materials under pH control no significant differences were observed among tested conditions, while at pH 7.60 organisms presented a significant decrease of their ETS activity at 0.10 mg/L Nf-MWCNTs in comparison to the remaining concentrations. For the individuals exposed to f-MWCNTs significant increase of the ETS activity was observed only at the highest concentration when maintained at pH control, while under acidified pH an opposite behaviour was observed, with a significant inhibition of ETS activity at 0.10 mg/L in comparison to the remaining concentrations (Fig. 1C).

When comparing specimens exposed to different MWCNTs at the same pH and exposure concentration, no significant differences between materials were observed (Table 5).

For each MWCNTs and exposure concentration, significant differences between pH levels were observed at 0.10 mg/L of both MWCNT materials, with lower ETS activity in both cases in individuals maintained at pH 7.60 in comparison to organisms under pH 8.00 (Fig. 1C).

#### 3.2.2. Indicators of oxidative stress

*3.2.2.1. Lipid peroxidation (LPO) levels.* Results of LPO levels in *H. diversicolor* showed that for Nf-MWCNT materials under pH control no significant differences were observed among all concentrations, while under pH 7.60 the levels significantly increased in contaminated organisms in comparison to non-contaminated ones. A similar trend was also observed in polychaetes exposed to f-MWCNTs under both pH levels, showing concentration-dependent increase of LPO levels in organisms exposed to the CNMs compared to control individuals (Fig. 2A).

When comparing individuals exposed to different MWCNTs at the pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.10 mg/L under both pH levels, with higher values in individuals contaminated with f-MWCNTs in comparison to organisms contaminated with Nf-MWCNTs (Table 5).

For each MWCNTs (f and Nf) and exposure concentration no differences were observed between pH levels (Fig. 2A).

*3.2.2.2. Catalase (CAT) activity.* Considering the effects of exposure concentrations, the results of CAT activity in organisms exposed to Nf-MWCNT materials at pH 8.00 showed no significant differences among concentrations, while a significant increase on the activity of this enzyme was observed in individuals exposed to 0.01 mg/L under pH 7.60 in comparison to uncontaminated individuals. Results of CAT activity in *H. diversicolor* exposed to f-MWCNT materials under pH 8.00 showed no significant differences among all concentrations, while a significant dose-dependent increase of the activity was recorded in organisms under pH 7.60 (Fig. 3A).

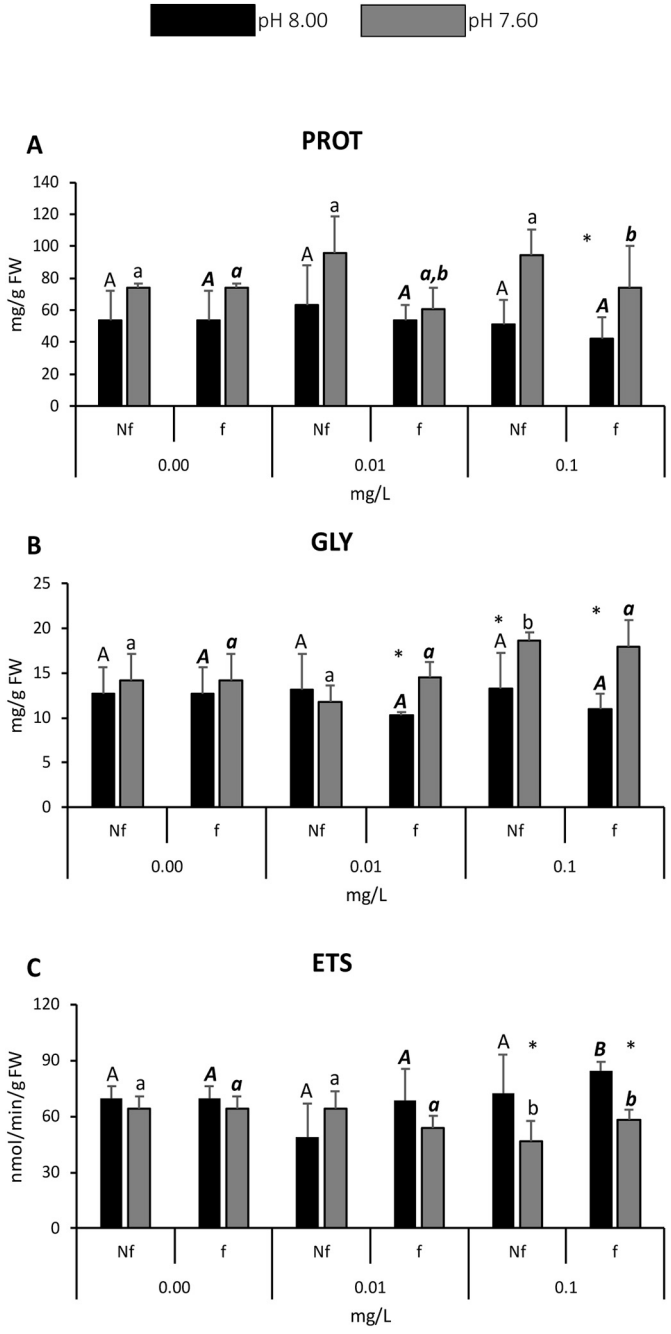


**Fig. 1.** A. Protein (PROT) content; B. Glycogen (GLY) content; C. Electron transport system (ETS) activity (mean + standard deviation) in *Hediste diversicolor* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (pH control-8.00; pH acidified-7.60). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNTs (f-MWCNTs and Nf-MWCNTs) and pH levels were represented with different letters: uppercase and regular letters for Nf-MWCNT at pH 8.00; lowercase and regular letters for Nf-MWCNTs pH 7.60; uppercase bold and italic letters for f-MWCNT at pH 8.00; lowercase, bold and italic letters for f-MWCNT at pH 7.60. Significant differences ($p \leq 0.05$) between the two pH levels for each MWCNTs and exposure concentration were represented with bold asterisks (*).

When comparing individuals exposed to different MWCNTs at the same pH and exposure concentration no significant differences between materials were observed (Table 5).

For each MWCNTs material and exposure concentration, significant differences between pH levels were observed in organisms exposed to 0.10 mg/L f-MWCNTs, showing higher CAT activity under pH 7.60 compared to individuals under pH 8.00 (Fig. 3A).

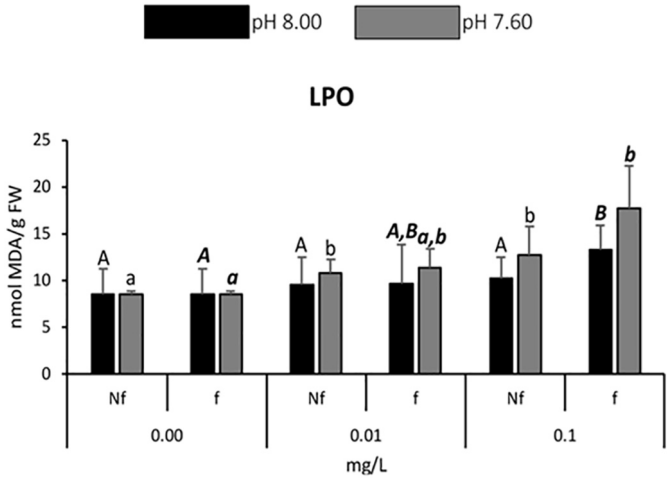


**Fig. 2.** Lipid peroxidation (LPO) levels (mean + standard deviation) in *Hediste diversicolor* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (pH control-8.00; pH acidified-7.60). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNTs (f-MWCNTs and Nf-MWCNTs) and pH levels were represented with different letters: uppercase and regular letters for Nf-MWCNT at pH 8.00; lowercase and regular letters for Nf-MWCNTs pH 7.60; uppercase bold and italic letters for f-MWCNT at pH 8.00; lowercase, bold and italic letters for f-MWCNT at pH 7.60. Significant differences ($p \leq 0.05$) between the two pH levels for each MWCNTs and exposure concentration were represented with bold asterisks (*).

*3.2.2.3. Superoxide dismutase (SOD) activity.* Along the increasing exposure concentrations of Nf-MWCNT materials, a concentration-dependent increase was observed in SOD activity for polychaetes under both pH levels in comparison to the non-exposed ones (Fig. 3B). In polychaetes contaminated with f-MWCNTs under pH 8.00, the SOD activity significantly increased at the highest exposure concentration (0.10 mg/L) in comparison to the other treatments, while under pH 7.60, the activity of this enzyme significantly increased in all the exposed individuals compared to non-exposed ones (Fig. 3B).

Comparing organisms under the same pH and exposure concentration, significantly higher SOD activity at 0.01 mg/L was observed in polychaetes exposed to Nf-MWCNTs compared to f-MWCNTs under pH 8.00 (Table 5).

For each MWCNTs (f and Nf) and exposure concentration no differences between pH levels were observed (Fig. 3B).

*3.2.2.4. Glutathione reductase (GR) activity.* Considering the effects of exposure concentrations, results of GR activity in *H. diversicolor* showed that for Nf-MWCNT materials under pH 8.00 no significant differences were observed among all conditions, while at pH 7.60 significantly higher activity was detected in exposed organisms in comparison to control ones. In polychaetes contaminated with f-MWCNTs under both pH levels GR activity showed no significant differences among concentrations (Fig. 3C).

When comparing specimens exposed to different MWCNTs at the same pH and exposure concentration, significantly higher GR activity at 0.01 mg/L was observed in organisms exposed to Nf-MWCNTs compared to f-MWCNTs under pH 8.00 (Table 5).

For each MWCNTs material at each exposure concentration, significant differences between pH levels were recorded in organisms exposed to 0.10 mg/L Nf-MWCNTs, with higher GR activity under pH 7.60 compared to individuals under pH 8.00 (Fig. 3C).

*3.2.2.5. Neurotoxicity*

*3.2.2.5.1. Acetylcholinesterase (AChE) activity.* Considering the effects of exposure concentrations, results of AChE activity showed significant differences among individuals exposed to different Nf-MWCNT concentrations under pH 8.00, with the lowest value recorded under 0.01 mg/L

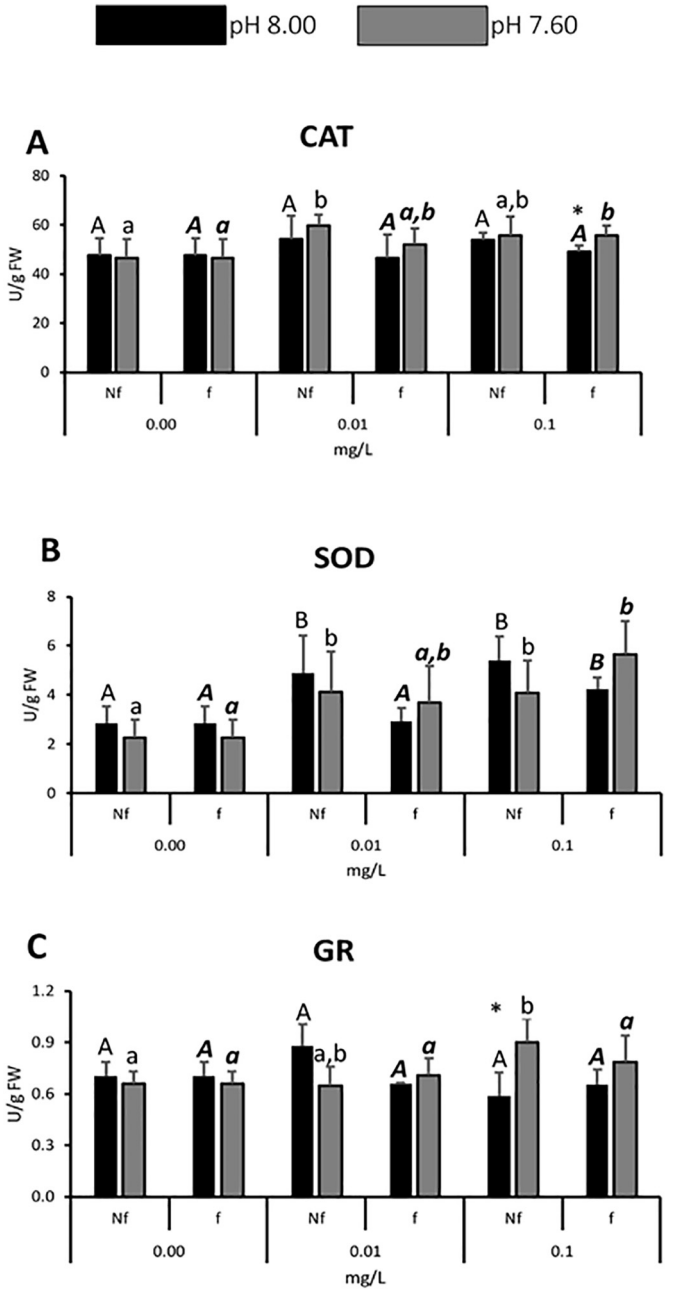


**Fig. 3.** A. Catalase (CAT) activity; B. Superoxide dismutase (SOD) activity; C. Glutathione reductase (GR) activity (mean + standard deviation) in *Hediste diversicolor* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (pH control-8.00; pH acidified-7.60). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNTs (f-MWCNTs and Nf-MWCNTs) and pH levels were represented with different letters: uppercase and regular letters for Nf-MWCNT at pH 8.00; lowercase and regular letters for Nf-MWCNTs pH 7.60; uppercase bold and italic letters for f-MWCNT at pH 8.00; lowercase, bold and italic letters for f-MWCNT at pH 7.60. Significant differences ($p \leq 0.05$) between the two pH levels for each MWCNTs and exposure concentration were represented with bold asterisks (*).

in comparison to the other conditions. Under pH 7.60 the activity of the neuro-enzyme was significantly lower only at 0.01 mg/L Nf-MWCNTs in comparison to the remaining conditions. Regarding the individuals contaminated with f-MWCNTs under both pH levels, significantly lower AChE activity was observed only in organisms exposed to 0.10 mg/L compared to all the remaining concentrations (Fig. 4).

When comparing organisms exposed to the same pH and exposure concentration, significant differences between polychaetes exposed to

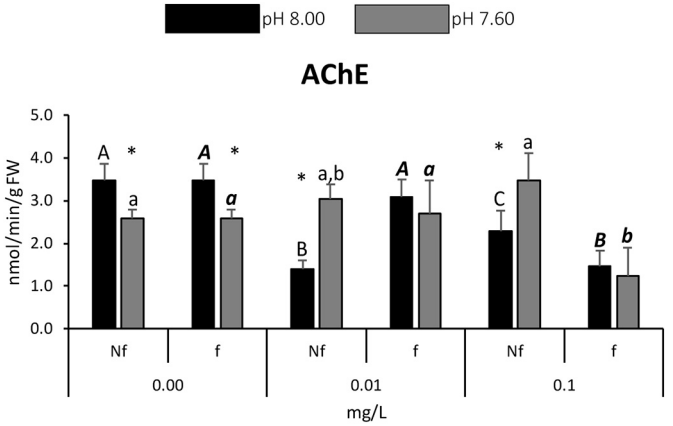


**Fig. 4.** Acetylcholinesterase (AChE) activity (mean + standard deviation) in *Hediste diversicolor* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (pH control-8.00; pH acidified-7.60). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNTs (f-MWCNTs and Nf-MWCNTs) and pH levels were represented with different letters: uppercase and regular letters for Nf-MWCNT at pH 8.00; lowercase and regular letters for Nf-MWCNTs pH 7.60; uppercase bold and italic letters for f-MWCNT at pH 8.00; lowercase, bold and italic letters for f-MWCNT at pH 7.60. Significant differences ($p \leq 0.05$) between the two pH levels for each MWCNTs and exposure concentration were represented with bold asterisks (*).

different MWCNTs were observed at 0.01 mg/L, with lower activity in *H. diversicolor* exposed to Nf-MWCNTs under pH 8.00 compared to individuals exposed to f-MWCNTs. Significant differences between materials were also observed in individuals exposed to 0.10 mg/L maintained under both pH levels, with lower activity in individuals contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 5).

For each MWCNTs material at each exposure concentration, differences between pH levels were observed between control individuals, showing the lowest AChE activity in organisms under pH 7.60. Differences between pH levels were also recorded in *H. diversicolor* exposed to 0.01 and 0.10 mg/L Nf-MWCNTs, with significantly lower enzyme activity in organisms mainteined under pH control compared to acidified pH (Fig. 4).

## 4. Discussion

In the present study we evaluated possible biochemical responses in terms of metabolism and energy reserves, oxidative and neuro status when *H. diversicolor* specimens were exposed to different environmental relevant concentrations of different CNMs under actual and predicted seawater acidification scenarios. Our findings showed that both CNMs under pH 7.60 generated greater toxic impacts in the polychaetes compared to individuals maintained under pH control and greater toxic impacts were caused by functionalized CNMs in comparison to the unfunctionalized ones, especially in terms of oxidative stress and neurotoxicity, due to their higher availability in the water media.

### 4.1. Energy reserves and metabolic capacity

Looking to polychaetes energy reserves and metabolism an opposite behaviour was observed for the individuals exposed to acidified pH and control pH. Under pH 7.60*H. diversicolor* exposed to both CNMs presented a dose-dependent decrease of electron transport system (ETS) activity and an increase of glycogen (GLY) and protein (PROT) contents. When organisms are submitted to stressful conditions oxidative stress may occur as a consequence of reactive oxygen species (ROS) over production, causing lipid peroxidation (LPO) of the mitochondria membranes, thus impairing the function of ETS activity (Bielen et al., 2016). This response could explain why in the presence of both CNMs at the highest exposure concentration the organisms showed a decrease of

metabolic rate preventing the consumption of energy reserves under acidified conditions. Similar results were observed by Sun et al. (2017). These authors conducted a 21-day experiment to investigate the impact of $CO_2$ enrichment-induced seawater acidification on key aspects of the haemocyte structure and immune function of the mussel *Mytilus edulis*. The organisms were exposed to pH levels mimicking near future ocean acidification (pH 7.7) showing that low pH negatively affected the efficiency of ETS by increasing the electron slip in the ROS-generating mitochondrial complexes I and III and/or by partially inhibiting the flow through the downstream ETS complexes. In fact, when *p*CO2 levels increase in seawater, dissolved $CO_2$ more readily diffuses across animal surfaces, crossing biological membranes and entering the intracellular spaces causing obvious impact on the physiological condition and functionality of the organisms (Fabry et al., 2008) and the suppression of metabolism is considered a "sublethal" reversible process, with the decrease of the energy reserves expressed as growth and reproductive output which effectively diminish the survival of the species on longer time-scales (Fabry et al., 2008). However, when *H. diversicolor* was exposed to pH control, a slightly increase of the metabolic capacity was observed under the highest concentration of f-MWCNTs. Nevertheless, no differences were observed when exposed to Nf-MWCNTs, which may indicate that under control pH the used concentrations were not high enough to result in metabolic depression. Moreover, the increase of ETS in the organisms exposed to f-MWCNTs at the highest concentration, could be due to the activation of defense mechanisms, as demonstrated under this exposure condition. Similar results were also obtained by Bertrand et al. (2016) which exposing the bivalve *Scrobicularia plana* to silver (Ag) NMs under cntrol pH conditions observed an increase of ETS activity indicating impairment of metabolic activity in clams that suffered from membranes cellular damage and activation of antioxidant enzymes. In this case, the observed behaviour of metabolic activity under control pH could be attributed to the surface functionalization of the CNTs. While raw CNTs do not readily cross biological barriers due to low dispersibility and low resident time in the water column, water dispersible MWCNTs (as for COOH-MWCNTs used in the present study), due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, induced higher levels of toxicity to biological systems (Arndt et al., 2013) causing higher cellular damage with the activation of antioxidant mechanisms (Freixa et al., 2018).

### 4.2. Oxidative stress

Besides metabolic alterations, the generation of ROS will also induce alteration on the oxidative balance of organisms (Bruno et al., 2009). Generally, under normal conditions ROS are regulated by enzymes including superoxide dismutase (SOD) and catalase (CAT), as well as by molecule natural antioxidants such as glutathione's (GPx and GR), ascorbic acid *etc.* However, if these mechanisms are not efficient in eliminating the excess of ROS produced a general oxidative stress status can occur, including oxidative damage to biomolecules as DNA, lipids, proteins and enzymes (Shvedova et al., 2012).

In the present study in the organisms exposed to Nf-MWCNTs under pH control the LPO did not increase along with the increasing exposure concentrations while under acidified pH the level was higher in the contaminated organisms in comparison to non-contaminated ones, clearly indicating higher impact of pH in polychaetes in comparison to the impacts caused by CNMs (probably due to low concentrations tested, low solubility and consequently low toxicity of non-functionalized MWCNTs or no effect of pH on CNMs toxicity). Looking to the results of f-MWCNTs the damage of the lipid membranes was observed under both pH conditions, assuming that all these different responses were directly related to the availability of the CNT materials as well as to the chemistry of the water media where the CNMs were dispersed since higher damage was observed under low pH indicating higher toxicity of CNMs at acidified conditions or, at the same time, higher sensitivity of polychates to CNMs under this condition. Considering the type of materials, while Nf-MWCNTs, due to their insolubility, as also demonstrated by DLS analysis, could be less available for the organisms, f-MWCNTs were more dispersible in the water column probably increasing their mobility and thus may intensify the risk of exposure and toxicity and possible uptake (Jackson et al., 2013). Regarding the exposure media, it is already known from the literature that the stability of CNMs in aquatic environment is significantly related to environmental factors, including pH, salinity, ionic strength and dissolved organic materials (Jackson et al., 2013). Changes in environmental conditions can affect the suspension of CNMs in seawater. Some studies have found that decreased pH can facilitate the dissolution of NMs in aquatic medium (Xia et al., 2018). Therefore, we hypothesized that ocean acidification could alleviate aggregation and agglomeration of the used CNMs, as also showed in the DLS analysis, which may increase the uptake and biodistribution into the organisms generating some synergistic and more toxic interactive effects of pH and CNMs on oxidative stress. In agreement with the present results, Huang et al. (2016) investigated the combined effects of low pH and nanoscale titanium dioxide (nano-$TiO_2$) in the mussel *Mytilus coruscus* showing that ROS increased with nano-$TiO_2$ concentrations under low pH conditions. All these results are in line with the physical/chemical behaviour of the NMs in presence of pH variations as confirmed by Jiang et al. (2009) who reported that the repulsive force of the NMs was weakened due to low surface charge and the hydrodynamic size increased when the pH approached the zero point of charge ($pH_{ZPC}$).

The generation of LPO is known to be responsible for the activation/inactivation of antioxidant enzymes, which play important roles in the total defense system against oxidative damage (Ighodaro and Akinloye, 2017). In the present study, CAT, SOD and GR showed similar antioxidant activities under both CNMs, however, significantly increased activities under acidified pH was observed especially at the highest exposure concentration of both materials. These results are in agreement with LPO levels described previously, suggesting an attempt by these enzymes to cope as compensatory response of cellular defense systems against cellular damage. Nevertheless, the excessive ROS production, especially under the highest exposure concentration, may lead to oxidative damage and a loss of compensatory mechanisms as a consequence of insufficient mechanism of the antioxidant activity (Walters et al., 2016; Fukai and Ushio-Fukai, 2011) which may contribute to higher LPO levels recorded under this condition. Again, this opposite behaviour could be associated with current exposure media rather than the type of nanoparticles. In agreement with the present results, also Huang et al. (2018) showed higher activities of antioxidant enzymes such as SOD, CAT and GPx in gills and hemocytes of the mussels *M. coruscus* when the organisms were subjected to low pH and high concentration of nanoparticulate zinc oxide (nano-ZnO) suggesting a major oxidative stress responses under the combination of the two stressors comparing the toxic effects caused by the NMs acting alone.

### 4.3. Neurotoxicity

The inhibition of the class of enzymes named cholinesterases (ChEs), esterases that hydrolyse mainly choline-based esters used as neurotransmitters, which the mechanism of action results in signs and symptoms of excessive cholinergic stimulation (Karami-Mohajeri and Abdollahi, 2011), has been already demonstrated when the invertebrates are exposed to different CNMs (De Marchi et al., 2017a, 2017b, 2017c, 2018a, 2018b, 2018c; Monserrat et al., 2017). In the present study different results were observed in the AChE activity depending on the type of materials. In fact, when organisms were exposed to Nf-MWCNTs under pH control the activity was inhibited especially under 0.01 mg/L while when polychaetes were submitted to acidified pH no neuro-inhibition was observed. Different behaviour was observed regarding f-MWCNTs, while under both pH levels the activity of the neurotrasmitter was lower only at the highest exposure concentration.

Looking on DLS analysis, the mean size of the f-MWCNTs was always lower in comparison to Nf-MWCNTs under both pHs respectively, which could help us to justify higher availability of the carboxylated form of MWCNTs also at the highest concentration for the organisms, intensifying the risk of exposure and possible absorption of the NMs, leading to a much higher neuro status damage in comparison to the isoluble form of MWCNTs. Such findings are in agreement with our previous studies that demonstrated neurotoxic capacity of functionalized CNMs in polychaetes (De Marchi et al., 2018a).

In conclusion, in what regards to polychaetes energy reserves and metabolism, in the presence of both CNMs the organisms prevented the consumption of energy reserves under acidified conditions, while when *H. diversicolor* was exposed to pH control the used concentrations were not high enough to result in metabolic depression. Looking at oxidative stress status, in organisms exposed to pristine CNMs under pH control, the LPO did not increase along with the increasing exposure concentrations while under acidified pH the level was higher in the contaminated organisms in comparison to non-contaminated ones, clearly indicating higher impact of pH in polychaetes in comparison to the impacts caused by CNMs despite the activation of antioxidant enzymes. The present results further demonstrated the neurotoxicity caused by both CNMs, especially noticeable at acidified conditions. Overall, we observed that the mechanism of enhanced toxicity in the exposed polychaetes should be attributed to slighter aggregation and more suspended CNMs in acidified seawater. Therefore, ocean acidification may cause a higher risk of CNMs to marine ecosystems due to increased sensitivity of polychaetes to CBMs.

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